

**TITLE OF THE INVENTION: Method for the screening of  $\alpha_2\delta$ -1 subunit binding ligands**

This application is a Continuation of USSN 09/397,549 filed September 16, 1999; the  
5 entire contents of which are herein incorporated by reference.

**FIELD OF THE INVENTION**

The invention relates to a method for the screening of ligands which bind a soluble secreted cerebral cortical voltage-dependent calcium channel  $\alpha_2\delta$ -1 subunit polypeptide.

10 **BACKGROUND OF THE INVENTION**

Gabapentin (1-aminoethyl-cyclohexane acetic acid) is currently commercialized for the treatment of epilepsy. The compound has however been recognized as being also useful for the treatment of pain and anxiety.

15 Recent reports have suggested an interaction between gabapentin and the  $\alpha_2\delta$  subunit of a voltage-dependent calcium channel (VDCC). But electro-physiological studies have yielded conflicting data on the action of gabapentin at VDCCs, even though the relevance of the interaction of gabapentin at the  $\alpha_2\delta$  subunit to the clinical utility of the drug is becoming clearer. However, none of the prototype anticonvulsant drugs displace  
20 [ $^3\text{H}$ ]gabapentin binding from the  $\alpha_2\delta$ -1 subunit.

The most frequently used assay currently available for the screening of ligands that bind the  $\alpha_2\delta$  subunit involves the use of pig membrane extracts as a source of the  $\alpha_2\delta$  subunit. Such an assay presents major inconvenience. Firstly, because the assay material is a  
25 membrane extract, it is very difficult to accurately determine the protein composition from one assay preparation to another particularly with regard to the subtype. Also, the presence of various impurities in the assay preparation is a problem in small plate assays. Furthermore, as the protein preparation lacks homogeneity, the interaction between the targeted protein and the assay plate is often quite uneven. This renders the streamlining of  
30 the assay in a high throughput format almost impossible to achieve.

Utility Application

EXPRESS MAIL NO. EJ054450643US  
A0000179-C1-66-MG

**SUMMARY OF THE INVENTION**

The inventors have found that it was possible to use a soluble secreted form of a voltage-  
5 dependant calcium channel  $\alpha_2\delta$ -1 subunit polypeptide (hereinafter  $\alpha_2\delta$ -1 subunit polypeptide) in an assay for the screening of ligands which bind the  $\alpha_2\delta$ -1 subunit.

The exact position and configuration of the [ $^3$ H]gabapentin binding site on the  $\alpha_2\delta$  subunit is not currently known. Furthermore, recent deletion experiments on the porcine  $\alpha_2\delta$ -1  
10 subunit coding sequence have shown that amino-acids close to the C-terminal region are needed in order for the protein to bind [ $^3$ H]gabapentin. For this very reason, the use of truncated forms of the porcine  $\alpha_2\delta$ -1 subunit in screening assays has not been disclosed or suggested in the prior art because there was concern as to whether relevant levels of binding capacity would be achieved in an assay environment.

15 The assay of the invention is of considerable interest because it confirms that a recombinant soluble secreted  $\alpha_2\delta$ -1 subunit polypeptide can be used in high throughput  $\alpha_2\delta$ -1 ligand screening. It also provides a useful advantage over the pig membrane extract screening assay as it allows the study of  $\alpha_2\delta$ -1 subtype-specific binding ligands. Proteins can be tagged which makes purifying convenient and possible to use a tagged antibody for  
20 recognition.

It was not clear whether the addition of the 6His tag to the C-terminus of the protein would affect the [ $^3$ H]gabapentin binding properties of  $\alpha_2\delta$

It was also unclear whether a C-terminally located 6His tag on  $\alpha_2\delta$  would be accessible for  
25 interaction with the Ni NTA chromatography matrix (for purification purposes) and SPA bead, or Ni flashplate well surface (for purposes of the assay).

The invention concerns a method for the screening of ligands which bind a calcium channel  $\alpha_2\delta$ -1 subunit.

30 The method comprises the steps of:

- contacting a secreted soluble recombinant calcium channel  $\alpha_2\delta$ -1 subunit polypeptide with:

- a ligand of interest; and
- a labelled compound which binds a  $\alpha_2\delta$ -1 subunit; and
- measuring the level of binding of the labelled compound to the secreted soluble  $\alpha_2\delta$ -1 subunit.

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The invention also concerns a kit for the screening of ligands which bind a calcium channel  $\alpha_2\delta$ -1 subunit.

The kit comprises:

- a secreted soluble recombinant calcium channel  $\alpha_2\delta$ -1 subunit polypeptide; and
- a labelled compound which binds a calcium channel  $\alpha_2\delta$ -1 subunit.

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#### **BRIEF DESCRIPTION OF THE FIGURES**

- 15 Figure 1 represents the elution profile of the recombinant polypeptide with the amino acid sequence of SEQ ID No 9 purified by Superdex-200 chromatography, either before or after electron on NI-NTA.

- Figure 2 illustrates the optimization of imidazole concentrations in an embodiment of the  
20 SPA assay of the invention.

Figure 3 illustrates the optimization of imidazole concentrations in an embodiment of the flashplate assay of the invention.

- 25 Figure 4 illustrates the flashplate time course of [ $^3\text{H}$ ]gabapentin binding to various concentrations of the recombinant polypeptide with the amino acid sequence of SEQ ID No 9.

- Figure 5 illustrates the capacity of the recombinant polypeptide with the amino acid  
30 sequence of SEQ ID No 9 in a flashplate assay after 3 hours of incubation.

Utility Application

Figure 6 illustrates the optimum imidazole concentration, assayed after 3 hours of incubation, required to maximize [ $^3\text{H}$ ]gabapentin binding using a constant amount of the recombinant polypeptide with the amino acid sequence of SEQ ID No 9.

- 5 Figure 7 illustrates flashplate assay of [ $^3\text{H}$ ]gabapentin saturation binding to the purified recombinant polypeptide with the amino acid sequence of SEQ ID No 9, assayed after 3 hours of incubation.

- Figure 8 illustrates the flashplate time course optimisation of imidazole concentration required to maximize the [ $^3\text{H}$ ]Leucine binding window to to the purified recombinant  
10 polypeptide with the amino acid sequence of SEQ ID No 9, assayed after 3 hours of incubation.

Figure 9 illustrates competition curves of three compounds in the flashplate assay format, assayed after 3 hours of incubation.

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#### **DETAILED DESCRIPTION OF THE INVENTION**

- The invention concerns a method for the screening of ligands which bind a soluble secreted  $\alpha_2\delta$ -1 subunit polypeptide. The term  $\alpha_2\delta$ -1 subunit polypeptide, when used herein, is  
20 intended to designate a structure containing two polypeptides ( $\alpha_2$  and  $\delta$ ) attached to one another by covalent disulfide bridges. More particularly, the targeted  $\alpha_2\delta$ -1 subunit binding site is preferably the [ $^3\text{H}$ ]gabapentin binding site. The various parameters of the method of the invention are described in further detail below.

#### **A – Secreted soluble recombinant $\alpha_2\delta$ -1 subunit polypeptide**

- Several nucleotide sequences encoding a secreted soluble form of an  $\alpha_2\delta$ -1 subunit can be used in the context of the present invention. Preferred soluble secreted  $\alpha_2\delta$ -1 subunit polypeptides are derived from eukaryotic  $\alpha_2\delta$ -1 subunits, more preferably from mammal, such as mouse, rat, rabbit, porcine, bovine or others and human  $\alpha_2\delta$ -1 subunits. Most  
30 preferred soluble secreted  $\alpha_2\delta$ -1 subunit polypeptides are derived from the human or porcine  $\alpha_2\delta$ -1 subunits.

Utility Application

More specifically, the selected nucleotide sequences encode a secreted soluble polypeptide having at least 80%, preferably 90%, more preferably 95%, and most preferably 98 or 99% amino-acid identity with the polypeptide comprising from amino acid 1 to between amino-acids 985 and 1054, preferably between amino-acids 985 and 1059, and most preferably between amino-acids 1019 and 1044 of SEQ ID NO:5 or SEQ ID NO:16.

In order to determine the optimal deletions on the  $\alpha_2\delta$ -1 subunit cDNA that yield a soluble secreted polypeptide devoid of membrane anchorage structures and having a functional [ $^3$ H]gabapentin binding site, the inventors tested the expression of several human or porcine  $\alpha_2\delta$ -1 subunit cDNA deletion mutants. The discussion provided below provides detailed comments on possible truncations, giving as an example the porcine  $\alpha_2\delta$ -1 subunit. However, given the very substantial cross-species homology for  $\alpha_2\delta$ -1 subunit sequences, the comments below can also be applied to other eukaryotic species, and more particularly other mammalian species such as the rat, the mouse or the rabbit. Their  $\alpha_2\delta$ -1 subunit sequences, which are available in public databases, share a very substantial homology with the human and porcine  $\alpha_2\delta$ -1 subunit sequences.

The inventors found that by deleting from the porcine  $\alpha_2\delta$ -1 subunit cDNA a nucleotide sequence encoding as much as amino-acids 967 to 1091 of the native protein, soluble polypeptides could be obtained. On the other hand, the minimal deletion required to achieve solubility appears to be located around nucleotides encoding amino-acids 1064 to 1091 of the sequence of SEQ ID NO:5. In this regard, the mutant polypeptide expressed using a cDNA deletion mutant from which a sequence encoding amino-acids 1064 to 1091 is removed is found in both soluble and membrane-associated forms, with [ $^3$ H]gabapentin and/or other derivatives or compounds such as pregabalin and gabapentoids binding properties similar to that of the wild type protein. Furthermore, a mutant protein expressed using a cDNA deletion mutant from which a nucleotide sequence encoding amino-acids 1085 to 1091 is removed recovers its membrane anchorage properties. Also, mutant proteins expressed using cDNA deletion mutants from which nucleotide sequences encoding either amino-acids 1037 to 1091 or amino-acids 1019 to 1091 of SEQ ID NO:5 or 16 are removed are found in soluble form.

The inventors believe that the soluble secreted  $\alpha_2\delta$ -1 subunit polypeptides which are as close as possible to the native sequence and which are therefore more likely to retain their native folding and hence their [ $^3\text{H}$ ]gabapentin- binding properties are those corresponding to a protein in which amino-acid stretch 985-1091 to 1074-1091, the amino-acid sequence of SEQ ID NO:5 or 16 has been deleted. The skilled scientist can quite easily determine within this amino-acid stretch the optimal mutant protein.

The invention therefore particularly concerns a screening assay in which the secreted soluble  $\alpha_2\delta$ -1 subunit polypeptide is preferably a polypeptide having at least 80% identity with the polypeptide comprising from amino-acid 1 to between amino-acid 985 and 1054, preferably between amino-acids 985 and 1059, and most preferably between amino-acids 1019 and 1064 of SEQ ID NO:5 or SEQ ID NO:16. Preferred  $\alpha_2\delta$ -1 subunit polypeptides which can be used in the present invention are those of SEQ ID N°6, 7, 8, 9, 13, 14 and 15, with the polypeptides of SEQ ID NO:9 or SEQ ID NO:15 being most preferred.

In a first and preferred embodiment of the invention, the  $\alpha_2\delta$ -1 subunit polypeptide is purified before it is used in the assay. The purification step, an example of which is provided further in this specification, can be carried out using several purification techniques well-known to the skilled person.

In some instances, it is required to tag the  $\alpha_2\delta$ -1 subunit polypeptide prior to purification. The tag is then in most instances encoded into the nucleotide sequence that is needed to express the polypeptide. Examples of such tags include, but are not limited to sequences encoding C-myc, FLAG, a sequence of histidine residues, heamagglutinin A, V5, Xpress or GST. Most of these tags can be incorporated directly into the sequence, for instance through PCR amplification by incorporating the appropriate coding sequence in one of the PCR amplification primers. However, the tag can also be introduced by other means such as covalent binding of the appropriate nucleic acid sequence encoding the tag moiety with the 5' or 3' end of the nucleic acid sequence encoding the polypeptide sequence. This is the

case for GST. It should be noted that the tag can be located at either end of the polypeptide sequence. Furthermore, in some instances, it can be advantageous to insert a cleavage site between the tag and the polypeptide sequence in order to permit removal of the tag sequence if needed.

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In other cases, providing a tag to the polypeptide is not needed. For instance, the protein can be purified using affinity columns loaded with specific monoclonal antibodies.

10 In a second embodiment of the invention, the  $\alpha_2\delta$ -1 subunit polypeptide can be only partially purified. For instance, it can be purified along with other contaminating proteins using an appropriate chromatography matrix such as ion-exchange chromatography column. In such instances, it is not required to tag the desired polypeptide of interest.

15 The most preferred embodiment contemplated by the inventors concerns the use of a purified tagged  $\alpha_2\delta$ -1 subunit polypeptide. A particularly preferred tag is a nucleotide sequence encoding from 2 to 10, and preferably 6 histidine residues as provided in the polypeptide of SEQ ID No 9.

20 With regard to the  $\alpha_2\delta$ -1 subunit polypeptide used subsequently in the screening assay of the invention, several possibilities are also open to the skilled person.

25 In a first and preferred embodiment, the  $\alpha_2\delta$ -1 subunit polypeptide comprises a tag moiety which can be selected among the tags referred to above. Such tagged polypeptides are particularly useful in SPA or flashplate assays. A preferred tag is the nucleotide sequence encoding histidine residues referred to above.

30 In a second embodiment, the  $\alpha_2\delta$ -1 subunit polypeptide can be used without a tag. This is the case for instance in SPA or flashplate assays comprising beads or plates coated with wheat germ lectin. In such an embodiment, the tag is not needed as the carbohydrate moieties of the  $\alpha_2\delta$ -1 subunit polypeptide bind directly to the wheat germ lectin-coated beads or plates.

**B - Labelled compounds which bind the  $\alpha_2\delta$ -1 subunit polypeptide**

In cases where the  $\alpha_2\delta$ -1 binding site is the [ $^3\text{H}$ ]gabapentin binding site, the preferred labelled compound which can be used is of course gabapentin itself. However, gabapentin is not the only labelled compound which can be used in this context. Indeed, it has been previously demonstrated that saturation binding analyses on porcine synaptic plasma cerebral cortex membranes performed in the presence of L-leucine indicate a competitive interaction of the amino acid with the [ $^3\text{H}$ ]gabapentin binding site, significantly reducing [ $^3\text{H}$ ]gabapentin binding affinity for the site. The inventors believe that this competitive interaction is true across across all the amino-acids listed in table 1 below.

**Table 1**

**Binding affinities of selected amino acids ( $\text{IC}_{50} < 500\text{nM}$ ) for the [ $^3\text{H}$ ]gabapentin site in porcine cortical membranes**

COMPOUND	$\text{IC}_{50}$ (NM) ARITHMETIC MEAN (N=3) $\pm$ S.E.M.
Gabapentin	42.1 $\pm$ 5.5
L-Norleucine	23.6 $\pm$ 6.7
20 L-Allo-Isoleucine	32.8 $\pm$ 6.0
L-Methionine	49.6 $\pm$ 10.0
L-Leucine	61.3 $\pm$ 20.9
L-Isoleucine	68.8 $\pm$ 1.9
L-Valine	330 $\pm$ 18
25 L-Phenylalanine	351 $\pm$ 89

It is therefore possible to use commercially available labelled forms of these high affinity ligands in replacement of gabapentin. The utility of [ $^3\text{H}$ ]L-leucine has been demonstrated in a filter binding assay and in a flashplate assay format. The inventors believe that labelled amino acids but also other compounds, with affinities preferably below 500 nM in the binding assay can be used as replacements of gabapentin.



With regard to the label, several embodiments can be used in the context of the invention. Preferred labels are of course radioactive labels, a list of which is provided further in this specification.

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### **C - Assay formats and conditions**

Several assay formats can be used to carry out the method of the present invention. Preferred assay formats include scintillation assays such as the scintillation proximity assay (SPA) or the flashplate assay. Other assay formats well known to those skilled in the arts such as the filter binding assay and the centrifugation assay are also contemplated in the present invention.

SPA and flashplate assays are preferred assay formats for the present invention. Additional details on these assays are provided below.

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### **Scintillation assay format**

Scintillation assays technology either involves the use of scintillant beads (for the SPA assay) or plates (for the flashplate assay). SPA beads are usually made from either cerium-doped yttrium ion silicate ( $\text{Y}_2\text{SiO}_5\text{:Ce}$ ) or polyvinyltoluene (PVT) containing an organic scintillant such as PPO. Flashplates commonly used are those such as Ni chelate flashplates although other flashplates can also be used.

Assays are usually carried out in aqueous buffers using radioisotopes such as  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$  or  $^{33}\text{P}$  that emit low-energy radiation, the energy of which is easily dissipated in an aqueous environment. For example, the electrons emitted by  $^3\text{H}$  have an average energy of only 6 keV and have a very short path length ( $\sim 1 \mu\text{m}$ ) in water. If a molecule labelled with one of these isotopes is bound to the bead or flashplate surface, either directly or via interaction with another molecule previously coupled to the bead or flashplate, the emitted radiation will activate the scintillant and produce light. The amount of light produced, which is proportional to the amount of labelled molecules bound to the beads, can be measured conveniently with a liquid scintillation (LS) counter. If the labelled molecule is

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not attached to the bead or a flashplate surface, its radiation energy is absorbed by the surrounding aqueous solvent before it reaches the bead, and no light is produced. Thus, bound ligands give a scintillation signal, but free ligands do not, and the need for a time-consuming separation step, characteristic of conventional radioligand binding assays, is eliminated. The manipulations required in the assays are reduced to a few simple pipetting steps leading to better precision and reproducibility.

The conditions under which SPA and flashplate assays are performed in the context of the present invention are provided below.

## 10 Scintillation assay conditions

### 1) SPA assay

The SPA assays is first developed to optimize the conditions under which the radioligand binds the  $\alpha_2\delta$ -1 subunit polypeptide. The parameters which can be varied to optimize radioligand binding in a typical SPA assay using Amersham beads include assay temperature,  $\alpha_2\delta$ -1 subunit polypeptide interaction with the radioligand and the SPA beads, radioligand concentration as well as pH variations.

The temperature at which the assay can be carried out can vary from 1 to 30°C. Preferred temperatures range from 18 to 23°C, with 21°C being the most preferred temperature. The interaction of the  $\alpha_2\delta$ -1 subunit polypeptide with the SPA beads can be optimized by adjusting the concentration of the polypeptide and by introducing a reagent which will favor this interaction. When 50 mg of Amersham SPA beads are used, the  $\alpha_2\delta$ -1 subunit polypeptide concentration may vary from 0.1 to 10 pmoles per well, with the optimal concentration being generally around 5 to 6 pmoles per well.

As for the reagent favoring the interaction between the  $\alpha_2\delta$ -1 subunit polypeptide and the radioligand as well as the Amersham SPA beads, the inventors found that imidazole could be efficiently used for that purpose when the  $\alpha_2\delta$ -1 subunit polypeptide is tagged with an amino acid sequence including 6 histidine residues. Furthermore, and more importantly, it was found that imidazole also enhanced binding of the radioligand to the  $\alpha_2\delta$ -1 polypeptide.

The optimal concentration of imidazole used to enhance radioligand binding varies depending on the concentration of  $\alpha_2\delta$ -1 subunit polypeptide used in the assay. For instance, when the concentration of the  $\alpha_2\delta$ -1 subunit polypeptide is about 20  $\mu$ l ( $\alpha_2\delta$ -1 polypeptide concentration of 0.6 pmol/ $\mu$ l), imidazole concentrations ranging from 10 to 50 mM can be used, with concentrations ranging between 10 and 30 mM being preferred. A most preferred imidazole concentration is 20 mM. It is to be noted that other compounds such as histidine can be used to enhance radioligand binding. Furthermore, pH variations can also influence radioligand binding although pH variations should be closely monitored as they may have an effect on the structural configuration of the  $\alpha_2\delta$ -1 subunit polypeptide. Also the use of imidazole is preferred to enhance radioligand binding, the person skilled in the art know that the use of imidazole is preferred but is absolutely not essential.

The concentration of the radioligand is evaluated with respect to the concentration of  $\alpha_2\delta$ -1 subunit polypeptide present in the assay medium. Generally, the concentration of radioligand varies from 1 nM to 100 nM. A preferred [ $^3$ H]gabapentin concentration is about 5 to 20 nM, with a most preferred concentration being about 10 nM. A preferred [ $^3$ H]leucine concentration is also about 5 to 20 nM, with a most preferred concentration being about 10 nM. It is to be noted that the concentration of other radioligands having affinities similar to those of [ $^3$ H]gabapentin and [ $^3$ H]leucine should also be in the range of about 5 to 20 nM.

Once the optimal radioligand binding conditions have been determined, a test ligand can be introduced in the assay medium to evaluate the level of displacement of the radioligand. The concentration of test ligand to be introduced in the assay medium usually varies from 0.1 nM to about 100  $\mu$ M. A preferred test ligand concentration of about 10  $\mu$ M is usually a starting point in a high throughput screening assay. Then, depending on the number of hits obtained, it may be lowered or increased.

It is to be noted that the parameters set forth above, which have been evaluated for a typical SPA assay using Amersham SPA beads can be adjusted by the skilled person, for example if SPA beads of a different type are used.

5    **2) Flashplate assay**

Similarly to the SPA assays, the flashplate can first be developed in order to optimize the conditions under which the radioligand binds the  $\alpha_2\delta$ -1 subunit polypeptide. The parameters which can be varied to optimize radioligand binding in a typical flashplate assay using NEN Ni chelate flashplates also include assay temperature,  $\alpha_2\delta$ -1 subunit  
10 polypeptide interaction with both the radioligand and the flashplates, radioligand concentration as well as pH variations.

The temperature at which the assay can be carried out can vary from 1 to 30°C. Preferred temperatures range from 18 to 23°C, with 21°C being the most preferred temperature.

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The interaction of the  $\alpha_2\delta$ -1 subunit polypeptide with the flashplates can be optimized by adjusting the concentration of the polypeptide and by introducing a reagent which will favor this interaction. When a standard NEN Ni chelate flashplate is used, the  $\alpha_2\delta$ -1 subunit polypeptide volume usually varies between 0.5 and 20  $\mu$ l for a concentration of  
20  $\alpha_2\delta$ -1 subunit polypeptide of 0.6 pmol/ $\mu$ l. As the published maximum binding capacity of NEN plates is about 6 pmol per well, the inventors consider that an optimal concentration of  $\alpha_2\delta$ -1 subunit polypeptide is probably around 5 pmol per well at 8 $\mu$ l.

Also the use of imidazole is preferred to enhance radioligand binding, the person skilled in  
25 the art know that the use of imidazole is preferred but is absolutely not essential.

With regard to the reagent favoring the interaction between the  $\alpha_2\delta$ -1 subunit polypeptide and the radioligand as well as the flashplates, the inventors found that imidazole could also be efficiently used for that purpose when the  $\alpha_2\delta$ -1 subunit polypeptide is tagged with an amino acid sequence including 6 histidine residues. It was also found that imidazole  
30 concentrations substantially enhanced binding of the radioligand to the  $\alpha_2\delta$ -1 polypeptide. The optimal concentration of imidazole used to enhance radioligand binding varies

depending on the concentration of  $\alpha_2\delta$ -1 subunit polypeptide used in the assay. For instance, when the volume of the  $\alpha_2\delta$ -1 subunit polypeptide is about 10  $\mu$ l (polypeptide concentration of 0.6 pmol/ $\mu$ l), the optimal imidazole concentration can vary between 1 and 20 mM, with a concentration of about 10 mM being preferred. As mentioned previously, other compounds such as histidine as well as pH variations may be used to enhance radioligand binding.

The concentration of the radioligand is evaluated with respect to the concentration of  $\alpha_2\delta$ -1 subunit polypeptide present in the assay medium. Generally, the concentration of radioligand varies from 1 nM to 100 nM. A preferred [ $^3$ H]gabapentin concentration is about 5 to 20 nM, with a most preferred concentration being about 10 nM. A preferred [ $^3$ H]leucine concentration is also about 5 to 20 nM, with a most preferred concentration being about 10 nM. It is to be noted that the concentration of other radioligands having affinities similar to those of [ $^3$ H]gabapentin and [ $^3$ H]leucine should also be in the range of about 5 to 20 nM.

Once the optimal radioligand binding conditions have been determined, a test ligand can be introduced in the assay medium to evaluate the level of displacement of the radioligand. The concentration of test ligand to be introduced in the assay medium usually varies from 0.1 nM to about 100  $\mu$ M. A preferred test ligand concentration of about 10  $\mu$ M is usually a starting point in a high throughput screening assay. Then, depending on the number of hits obtained, it may be lowered or increased.

The inventors have tested the displacement of a particular radioligand, [ $^3$ H]gabapentin, with (S+)-3-isobutyl gaba, (R-)-3-isobutyl gaba and gabapentin. The data provided in the examples which follow clearly shows that the assay can be used in high throughput competition studies.

**Example 1****Construction of a nucleotide sequence encoding the putative soluble porcine  $\alpha_2\delta$ -1b deletion mutant of SEQ ID NO:9****a) Primer design**

- 5 PCR primers were designed to generate the soluble porcine  $\alpha_2\delta$ -1b deletion mutant of SEQ ID NO:9 as follows:

5' PCR primer: This was designed to engineer in a KOZAK translation initiation consensus sequence prior to the coding sequence (Kozak *JBC* **266** 19867-19870)

- 3' PCR primer: This was designed to engineer in six histidine residues followed by a stop-codon at the desired location in the coding sequence. In addition to the stop codon the  $\alpha_2\delta$ -1 primers also included an *Eco* RI restriction site.

The bold region in each primer sequence denotes the 'tagged' region; addition of sequences not present in the template. Primers were custom synthesized by Perkin Elmer Applied Biosystems UK to the ABI ready pure grade, supplied lyophilized then resuspended to 15  $\mu$ M in 10mM TE. JB189 and 195 were provided without 5' phosphate groups:

5' primer JB189 (5' -TCGCCACCATGGCTGCTGGCTGCCTGCTG-3' , SEQ ID NO:20)

- 20 3' primer JB195 (5' -TCGGAATTCCTCAGTGATGGTGATGGTGATGAGAAACACCACCACAGTCGGT-3' , SEQ ID NO:21)

**b) PCR protocols for the generation of the  $\alpha_2\delta$ -1 deletion mutant**

- 25 **1) Generation of the pcDNA3-porcine- $\alpha_2\delta$ -(+) PCR template**

An oligo dT-primed  $\lambda$ gt10 porcine cerebral cortical cDNA library was screened by ECL (Amersham) using a 2,381-bp *HindIII* fragment (coding sequence 268-2649) of the rabbit skeletal muscle  $\alpha_2\delta$  clone (pcDNA3-Rab- $\alpha_2\delta$ -(+) (supplied by Neurex) as the probe.

- A positive insert was identified and subcloned into pBluescript-SK-(+) to generate pB-PC- $\alpha_2\delta$ -1.1. The clone was sequenced on both strands, except for a 711-bp stretch at one end of the clone, which had a high degree of homology to mitochondrial C oxidase.

The  $\alpha_2\delta$  coding region was homologous to the 3' region of the human neuronal  $\alpha_2\delta$  sequence but lacked 926 bp of 5' coding sequence. The missing sequence was obtained by 5'-RACE using total RNA prepared from porcine cerebral cortex. RACE was performed across a *Bgl* I site unique in known  $\alpha_2\delta$  sequences (rabbit (accession no. M21948)), rat  
5 (accession number M86621), human (accession no. M76559)

The sequence derived from the 5' RACE product was used to design a primer (JB042, 5'-GGGGATTGATCTTCGATCGCG-3'; SEQ ID NO:18) specific for the 5'-untranslated end of the cDNA. PCR was then performed with *Pfu* DNA polymerase using JB042 and a primer downstream of the *Bgl* I site (JB040, CTGAGATTTGGGGTTCTTTGG, SEQ ID  
10 NO:19).

The PCR product was ligated to Eco RI linkers (5'-GGAATTCC-3') and then digested with Eco RI and *Bgl* I. The 1,564-bp fragment (5' portion of the  $\alpha_2\delta$  cDNA) was gel-purified.

15 Similarly, a 2,303-bp fragment (3' portion of the  $\alpha_2\delta$  cDNA) was isolated after digestion of pB-PC- $\alpha_2\delta$ -1.1 with *Bgl* I and Eco RI. The two fragments of  $\alpha_2\delta$  cDNA were then ligated to EcoRI-digested pcDNA3 in a three-way ligation. A clone was picked with the full-length  $\alpha_2\delta$  sequence in the positive orientation with respect to the cytomegalovirus promoter (pcDNA3-PC- $\alpha_2\delta$ -(+)).

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## **2) PCR protocol**

The following reagents were added to obtain two cocktails labelled 'lower' and 'upper' buffers.

	<i>Lower</i>	$\mu$ l
25	10x <i>Pfu</i> DNA polymerase buffer	25
	10mM dNTP's	5
	100ng/ $\mu$ l pcDNA3-porcine- $\alpha_2\delta$ -(+)	10
	15 $\mu$ M JB189	8.5
	15 $\mu$ M JB195	8.5
30	H <sub>2</sub> O	193

<i>Upper</i>	$\mu$ l
10x <i>Pfu</i> DNA polymerase buffer	25
H <sub>2</sub> O	220
2.5units/ $\mu$ l <i>Pfu</i> DNA polymerase	5

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50 $\mu$ l aliquots of lower buffer were added to each of four 0.5ml eppendorf tubes. To each was added one PCRgem 100 ampliwx bead (PE biosystems). Tubes were heated to 80°C for 2 minutes then cooled to 4°C. 50 $\mu$ l of upper buffer was then added to each tube. Tubes were then cycled on a Stratagene Robo-Cycler according to the following conditions: 98°C / 1min 30sec, followed by: for 20 cycles 98°C / 45sec, 54°C / 2min, 72°C / 6min, followed by: 72°C / 20min, followed by: hold at 4°C.

The 3228bp PCR product was then purified on a QIAquick PCR purification column (Qiagen) and eluted with 61 $\mu$ l of H<sub>2</sub>O. The following reagents were added to the eluted

15 DNA: 0.7 $\mu$ l 10mM ATP, 7 $\mu$ l 10x Polynucleotide Kinase buffer, 1 $\mu$ l 1unit/ $\mu$ l Polynucleotide Kinase.

The above 5' phosphorylation reaction was incubated at 37°C for 1 hour. The reaction was stopped by incubation at 65°C for 10min. The 3228bp 5' phosphorylated PCR product was

20 then gel purified from a 1% agarose gel using QIAEX (Qiagen) beads and eluted in ~50 $\mu$ l.

## **Example 2**

### **Cloning of the PCR fragments of Example 1 into the Baculovirus transfer vector pFastBac1**

25 The PCR products of Example 1 (3228bp JB189/JB195 derived PCR product coding for 6His tagged porcine  $\alpha_2\delta$ -1b: SEQ ID No 9) were cloned into *Stu* I digested, calf intestinal phosphatase dephosphorylated, phenol chloroform extracted and QIAEX gel purified pFastBac1 (Life Technologies) using the Rapid DNA ligation kit (Roche Diagnostics) transforming XL1-blue ( $\alpha_2\delta$ -1b) *E. Coli* cells:

30



**a) Screening for positive recombinants**

Given that the PCR product was cloned by blunt-end ligation a screen was required to select a recombinant with the gene ligated in the positive orientation with respect to the polyhedrin promoter in pFastBac1. This was achieved by restriction digest of miniprep

- 5 DNA (Qiagen miniprep kit) prepared from colony minicultures and analysis on a 1% TAE agarose gel. A positive clone was identified according to the following digest patterns:

SEQ ID No 9 in pFastBac1

*Eco* RI digest performed on miniprep DNA

10	Predicted fragments (bp)
PCR product cloned in a positive orientation	4773 and 3230
PCR product cloned in a negative orientation	7989 and 14

**b) Sequencing analysis of selected clones**

- 15 One positive was selected for this clone and used to prepare a plasmid DNA stock of the desired construct (QIAGEN maxi kit). Confirmatory sequence reactions were performed using the Big Dye terminator sequencing kit and run on an ABI 310 Prism Genetic Analyzer. Sequence analysis of both coding strands was performed using a selection of sequencing oligonucleotide primers and has yielded the following results:

- 20 Sequencing of pFBac-Porcine-s- $\alpha_2\delta$ -1- $\Delta$ 1040-1067-6His confirmed that the insert sequence corresponded to the nucleic acid encoding the polypeptide of SEQ ID No 9, except for the deletion of two bases from the 5' end of the 5' PCR primer (JB189). The loss of these two bases did not have any impact on the 5' end of the gene as the KOZAK
- 25 translation start-site consensus sequence (GCCACC) starts immediately after this deletion.

**Example 3****Protocol for establishing baculovirus banks for the expression of the  $\alpha_2\delta$ -1 deletion mutant of SEQ ID NO:9**

- 30 Essentially, the protocol used to generate the baculovirus banks is that outlined in the Life Technologies Bac-to Bac<sup>TM</sup> baculovirus expression systems manual.

**a) Transposition of DH10Bac *E Coli* cells**

One ng (5µl) of the recombinant pFastBac-1 construct containing the nucleotide sequence encoding the porcine  $\alpha_2\delta$ -1 deletion mutant of SEQ ID No 9 was added to 100µl of

- 5 DH10Bac cells thawed on ice. The cells were then mixed gently by tapping the tube then incubated on ice for 30minutes before heat shock treatment by incubation in a 42°C water bath for 45 seconds. The mixture was then chilled on ice for 2 minutes before the addition of 900µl of S.O.C. medium. The mixture was then placed in a shaking incubator (200rpm) at 37°C for 4hours. The cells were then serially diluted (10 fold dilutions from  $10^{-1}$  to  $10^{-3}$ )
- 10 and 10µl of each dilution plated on LB agar plates containing 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline, 100µg/ml Bluo-gal and 40µg/ml IPTG. The plates were incubated at 37°C for between 1 and 3 days until discrete colonies of blue and white colour were discernible.

15 **b) Isolation of recombinant DNA**

- White colonies (containing the recombinant bacmid) were picked and grown for 24 hours (to stationary phase) at 37°C with shaking (200rpm) in 2ml of LB containing 50µg/ml kanamycin, 7µg/ml gentamicin and 10µg/ml tetracycline. 1.5ml of culture was then transferred to a microfuge tube and centrifuged at 14,000xg for 1minute. The supernatant
- 20 was removed and the cells resuspended gently in 0.3ml of 15mM Tris-HCl (pH8.0), 10mM EDTA, 100µg/ml RNase A. 0.3ml of 0.2N NaOH, 1% SDS was then added and the mixture mixed gently before incubation at 22°C for 5 minutes. Then 0.3ml of 3M potassium acetate (pH5.5) was added and the sample placed on ice for 10 minutes. After centrifugation at 14,000xg for 10 minutes the supernatant was transferred to a tube
- 25 containing 0.8ml of isopropanol, mixed then placed on ice for 10 minutes before centrifugation at 14,000xg for 10 minutes. The supernatant was then discarded and the pellet rinsed with 0.5ml of 70% ethanol before centrifugation at 14,000xg for 5 minutes. This 70% ethanol rinse was then repeated before removing all of the supernatant and air drying the pellet for 10 minutes at room temperature. The pellet was finally resuspended in
- 30 40µl of TE.

**c) Transfection of sf9 cells with the recombinant bacmid DNA**

- A 6-well tissue culture plate was seeded with  $0.9 \times 10^6$  sf9 cells (cells at log phase having grown from a culture passaged at  $0.3 \times 10^6$  cells/ml) per 35mm well in 2ml of Sf-900 II SFM media containing 50units/ml penicillin and 50 $\mu$ g/ml streptomycin. Cells were left to
- 5 attach at 27°C for 1 hour. Bacmid DNA prepared as described above (5 $\mu$ l) was added to 200 $\mu$ l of Sf-900 II SFM media containing 6 $\mu$ l of CELLFECTIN and mixed before incubation at room temperature for 45 minutes. The cells were washed once with 2ml of Sf-900 II SFM media without antibiotics then 0.8ml of Sf-900 II SFM media was added to each tube containing the lipid-DNA complex. The wash buffer was removed from the cells
- 10 and the 1ml of diluted lipid-DNA complex overlaid on the cells. The cells were incubated for 5hours at 27°C after which time the transfection mixture was removed and 2ml of Sf-900 II SFM media containing 50units/ml penicillin and 50 $\mu$ g/ml streptomycin was added. The cells were then incubated for 72 hours.
- 15 After incubation for 72 hours the media was removed from the cells and centrifuged at 500xg for 5 minutes. The supernatant was then transferred to a fresh tube, this was labelled as the P0 bank and stored at 4°C in the dark. The P1 bank was prepared by passaging sf9 cells at approx  $5 \times 10^6$  cells/ml to  $2 \times 10^6$  cells/ml (100ml in a 250ml Erlenmeyer flask) and adding 0.5ml of the P0 bank harvested above. The cells were then incubated shaking
- 20 (200rpm) at 27°C for 4 days. Under sterile conditions the culture was centrifuged at 500xg for 10 minutes and the supernatant 0.2 $\mu$ M filtered (P1 bank). The P2 bank was prepared by adding 2ml of P1 bank per 400ml culture (in 1L Erlenmeyer flasks) passaged as above to  $2 \times 10^6$  cells/ml. The culture was incubated as before for 4 days and the supernatant harvested and filtered as described for the P1 bank. The supernatant was first pooled then
- 25 aliquoted (10ml) and stored at 4°C.

**Example 4****Protein expression**

- To sf9 cells passaged from  $\sim 5 \times 10^6$  cells/ml to  $2 \times 10^6$  cells/ml in Sf-900 II SFM media was
- 30 added 0.1ml virus per 100 ml of cells of the appropriate viral bank (400ml volumes in 1L Erlenmeyer flasks). The cells were then cultured for 4-5 days at 27°C with 110 rpm

shaking. Expression of the protein was confirmed by SDS-PAGE and Western blotting using an anti penta-His monoclonal antibody (Qiagen) and was detected in the culture supernatant and cell lysate.

## 5 **Example 5**

### **Purification of $\alpha_2\delta$ -1 deletion mutant of SEQ ID NO:9**

The  $\alpha_2\delta$ -1 deletion mutant of SEQ ID NO:9 was purified from the cell lysate following the purification strategy outlined below:

The culture was centrifuged at 6,000xg for 10 minutes and the supernatant removed. The weight of the cell pellet was determined before re-suspension in 20mM Tris pH8.0, 100mMKCl, 1% P40-Nonidet (100ml per 20g of wet cells). A protease inhibitor cocktail (Sigma Cat# P8849), 1ml/L, was added to the mixture. The solution was then stirred for 10 minutes before centrifugation for 1hour at 30,000xg and 4°C. The supernatant was concentrated (30kDa cut off) to approx. ~300ml then centrifuged for 1hour at 100,000xg.

Supernatant containing the soluble proteins was diluted 1:3 in 10mM Tris-HCl pH8.0 (equilibration buffer) and loaded onto a pre-equilibrated Q-Sepharose column (2.5cm i.d. x 30cm h.) at a flow rate of 900ml/h. After washing with equilibration buffer until a stable  $A_{280nm}$  baseline had been achieved, protein was eluted with 20mM Tris-HCl pH8.0, 0.5M KCl, 10mM Imidazole.

The eluate was then loaded onto a Ni-NTA (Qiagen) column (2.5cm i.d. x 6cm h.) pre-equilibrated in 20mM Tris pH8.0, 0.5M KCl, 10mM Imidazole at a flow rate of 2 ml/min. The column was washed successively with buffer A (20mM Tris pH8.0, 0.5M KCl, 20mM Imidazole), buffer B (100mM Tris-HCl pH8.0, 1M KCl), and buffer A again. Elution was performed with buffer C (20mM Tris-HCl pH8.0, 100mM KCl, 0.5M Imidazole). The Ni-NTA eluate (~50ml) was concentrated (30kDa cut-off) to ~2ml and applied at 1ml/min and in 0.2ml aliquots, to an FPLC Superdex-200 column equilibrated in 10mM HEPES, pH7.4, 150mM NaCl. Fractions containing the polypeptide of SEQ ID No 9 were pooled. As shown in Figure 1, the protein elution profile and associated [ $^3$ H]gabapentin binding activity is presented together with a silver-stained SDS-PAGE gel (post Ni NTA load of

Superdex-200) demonstrating the co-elution of the ~130kDa band ( $\alpha_2\delta$ ) with the [ $^3\text{H}$ ]gabapentin binding activity and  $A_{280\text{nm}}$  profile.

### **Example 6**

#### **5 SPA assay of [ $^3\text{H}$ ]gabapentin binding to soluble porcine $\alpha_2\delta$ -1b-6His**

The assay was carried out at 21°C. Assay components were added in the following order (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C) to 96-well Optiplates:

- 25µl imidazole at various concentrations (diluted from a 1M stock pH8.0, see assay details)
- 10 50µl 10mM HEPES pH 7.4
- 25µl (50mg) SPA beads (Amersham)
- 100µl s- $\alpha_2\delta$ -1b-6His of SEQ ID No 9 (2µl protein diluted to 100µl) obtained from example 5
- 25µl radioligand ([ $^3\text{H}$ ]gabapentin )

- 15 Immediately after adding radioligand, the optiplates were loaded in the Packard Top Count scintillation counter to follow the binding time course. Imidazole was first used in the assay to optimize the specific interaction of the protein's 6His tag with the SPA bead. Imidazole itself (up to 100mM) in the filtration assay has no effect on [ $^3\text{H}$ ]gabapentin binding (n=1).

- 20 As shown in figure 2, specific binding of [ $^3\text{H}$ ]gabapentin to the s- $\alpha_2\delta$ -1b-6His was enhanced by imidazole. Of the concentrations, tested the optimal was 50mM. Equilibration was reached after ~2hours.

#### **25 Example 7**

#### **Ni Flashplate assay of [ $^3\text{H}$ ]gabapentin binding to soluble porcine $\alpha_2\delta$ -1b-6His (SEQ ID No 9)**

- Assays were carried out at 21°C in a final volume of 250µl in 96-well NEN Ni chelate flash plates. Assay components were added in the following order (all reagents were
- 30 diluted in 10mM HEPES (pH 7.4 at 21°C)):

25µl 10mM HEPES pH7.4

25µl imidazole at various concentrations (diluted from a 1M stock  
pH8.0, see assay details)

75µl 10mM HEPES pH 7.4

100µl s- $\alpha_2\delta$ -1b-6His (2µl protein diluted to 100µl) obtained from  
example 5

25µl radioligand ([ $^3$ H]gabapentin)

Immediately after adding the radioligand, flash plates were loaded in the Packard Top  
Count scintillation counter to follow the binding time course. The '[ $^3$ H] flash plate'  
programme (cpm) was used to monitor activity. Imidazole was first used in the assay to  
optimize the specific interaction of the protein's 6His tag with the Ni flashplate. Imidazole  
itself (up to 100mM) in the filtration assay has no effect on [ $^3$ H]gabapentin binding (n=1).

As shown in figure 3, the specific binding of [ $^3$ H]gabapentin to the s- $\alpha_2\delta$ -1b-6His was  
enhanced by imidazole. Initially, from the concentrations tested, the best concentration was  
found to be 10mM.

Specific binding was determined at different volumes of s- $\alpha_2\delta$ -1b-6His, in the presence of  
10mM imidazole, over a time period of 10h. Results are shown in figure 4 and equilibrium  
was reached at ~3h. Specific binding increased linearly with increasing amounts of protein,  
up to 8µl, after which the binding capacity of the Ni chelate in the assay well was probably  
exceeded (see figure 5). The published maximum binding capacity of NEN plates is  
6pmol/well. The concentration of purified s- $\alpha_2\delta$ -1b-6His is estimated at ~0.6pmol/µl,  
which yields 5pmol/well at 8µl.

## Table 2

### Saturation studies

Saturation experiments were performed with 12 duplicate data points, [ $^3$ H]gabapentin  
concentration ranged from ~1 to 350nM. Data was analyzed using KEL-RADLIG for  
Windows.

<b><u>Flash plate</u></b> (2µl protein used, n=2)	<b><u>Filter binding</u></b> K <sub>D</sub> (nm) (4µl protein used, n=3)
K <sub>D</sub> , 9.32nM K <sub>D</sub> , 10.5nM  <b>Mean = 9.91nM</b>	K <sub>D</sub> , 12.3nM K <sub>D</sub> , 8.91nM K <sub>D</sub> , 10.6nM  <b>Mean = 10.60 ± 0.98nM</b>

**Example 8****Ni Flashplate assay of [<sup>3</sup>H]Leucine binding to soluble porcine  $\alpha_2\delta$ -1b-6His**

- The procedure described in example 2 was repeated, except that [<sup>3</sup>H]gabapentin was replaced by 25  $\mu$ l (10.1 nM) of [<sup>3</sup>H]Leucine, as shown in figure 8, [<sup>3</sup>H]Leucine binds with
- 5 high affinity to soluble  $\alpha_2\delta$ -1b-6His. This demonstrates that it is possible to use commercially available forms of [<sup>3</sup>H]Leucine in replacement of [<sup>3</sup>H]gabapentin in the assay.

**Example 9**

10 **Ni Flashplate assay studying competitive binding of [<sup>3</sup>H]gabapentin, (S+)-3-isobutyl GABA and (R-)-3-isobutyl GABA to porcine  $\alpha_2\delta$ -1b-6His (SEQ ID No 9)**

- Assays were carried out at 21°C in a final volume of 250 $\mu$ l in 96-well NEN Ni chelate flash plates. Wells were set up for both 'total' and 'non-specific' binding. Specific binding
- 15 was defined as that remaining after subtraction of the average of the 'non-specific binding' values from the average of the 'total' binding values. Assay components were added in the following order (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C)):

	25 $\mu$ l	10mM HEPES pH7.4 or 25 $\mu$ l of the test compound at the appropriate concentration in HEPES
20	25 $\mu$ l	200 mM imidazole (diluted from a 1M stock pH8.0, see assay details)
	Total binding	75 $\mu$ l 10mM HEPES pH 7.4
	Non-specific binding	50 $\mu$ l 10mM HEPES pH 7.4 and 25 $\mu$ l 100 $\mu$ M (S+)-3-isobutyl GABA
25		100 $\mu$ l s- $\alpha_2\delta$ -1b-6His (2 $\mu$ l protein* diluted to 100 $\mu$ l)
		25 $\mu$ l radioligand ([ <sup>3</sup> H]gabapentin or [ <sup>3</sup> H]Leucine)

- \* The source of s- $\alpha_2\delta$ -1b-6His was that purified by FPLC Superdex-200 gel filtration (see example 5)
- 30 Immediately after adding radioligand, flash plates were loaded in the Packard Top Count scintillation counter to follow the binding time course. Incubation time before the assay



was 3 hours. The '[<sup>3</sup>H] flash plate' programme (cpm) was used to monitor activity. Specific binding was ~98% of the 'total' value. Imidazole was used in the assay to optimize the specific interaction of the protein's 6His tag with the Ni flashplate. Imidazole itself (up to 100mM) in the filtration assay has no effect on [<sup>3</sup>H]gabapentin binding (n=1).

5

Competition studies were compared across the flash-plate and filter binding methodologies in order to validate the new assay technology with the established filter binding methodology.

- 10 GraphPad Prism software was used to process competition curve data and determine IC<sub>50</sub> and hill slope values. Twelve point competition curves with half log dilution steps of test compounds were used in the experiments. Results are shown in Table 3 below where IC<sub>50</sub> values are presented, and in figure 9 where hill slopes range from -0.9 to 1.3. The [<sup>3</sup>H]Gabapentin concentration used in assay is in the range of 10-13nM

15

### Table 3

#### Competition studies:

GraphPad Prism software was used to process competition curve data and determine IC<sub>50</sub> and hill slope values. Ten point competition curves with half log dilution steps of test compounds were used in the experiments.

20

IC<sub>50</sub> values were converted to Ki values (presented in table) according to the following equation:

$$K_i = IC_{50} / (1 + [L]/K_D)$$

The K<sub>D</sub> values used were those mean values presented in table 1.

- 25 The [<sup>3</sup>H]Gabapentin concentration in the assay ranged from 10-13nM and was determined for each experiment for the purpose of calculating the Ki value as described above.

Hill slopes were all in the range of -0.9 to 1.3

<u>Test compound</u>	<u>Flash plate</u> (3µl protein used, n=2)	<u>Filter binding</u> <u>K<sub>D</sub>(nm)</u> (4µl protein used, n=3)
Gabapentin	10.4 7.97	7.13 7.70 10.2
<b>Mean (geometric)</b>	<b>9.10nM</b>	<b>7.84nM</b>
(S+)-3-isobutyl GABA	10.9 7.58	6.52 6.21 8.29
<b>Mean (geometric)</b>	<b>9.09nM</b>	<b>6.95nM</b>
(R-)-3-isobutyl GABA	157 207	78.4 64.2 107
<b>Mean (geometric)</b>	<b>180nM</b>	<b>81.5nM</b>

#### 5 Example 10

##### Filter binding assay of [<sup>3</sup>H]gabapentin binding to the recombinant polypeptide of SEQ ID No 9

Assays were carried out at 21°C in a final volume of 250µl in 96-deep well plates. Assay

- 10 components were (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C)):

25µl compound to test

200µl Polypeptide of SEQ ID No 9 (3µl protein diluted to 200µl)

25µl radioligand ([<sup>3</sup>H]gabapentin (65Ci/mmol))

- 15 Plates were incubated at room temperature for 1h prior to filtering on to 96-well GF/B Unifilter plates pre-soaked in 0.3% polyethylenimine. Filters were washed with 3x1ml 50mM Tris-HCl (pH 7.4 at 4°C), and dried over-night. Scintillant (Microscint O, 50µl) was added and the plates counted using a Packard Top Count scintillation counter. Specific binding was ~98% of the 'total' value. In [<sup>3</sup>H]gabapentin saturation studies, the K<sub>D</sub> (nM)
- 20 obtained was about 10.62.

Utility Application

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